Juvenile hormone regulates body size and perturbs insulin signaling in *Drosophila*

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The role of juvenile hormone (JH) in regulating the timing and nature of insect molts is well-established. Increasing evidence suggests that JH is also involved in regulating final insect size. Here we elucidate the developmental mechanism through which JH regulates body size in developing *Drosophila* larvae by genetically ablating the JH-producing organ, the corpora allata (CA). We found that larvae that lack CA pupariated at smaller sizes than control larvae due to a reduced larval growth rate. Neither the timing of the metamorphic molt nor the duration of larval growth was affected by the loss of JH. Further, we show that the effects of JH on growth rate are dependent on the forkhead box O transcription factor (FOXO), which is negatively regulated by the insulin-signaling pathway. Larvae that lacked the CA had elevated levels of FOXO activity, whereas a loss-of-function mutation of FOXO rescued the effects of CA ablation on final body size. Finally, the effect of JH on growth appears to be mediated, at least in part, via ecdysone synthesis in the prothoracic gland. These results indicate a role of JH in regulating growth rate via the ecdysone- and insulin-signaling pathways.

size control | developmental hormones | insect physiology

To correctly regulate their body size, animals control both the rate and duration of their growth. Canonically, growth rate and growth duration have been thought of as separate processes regulated by independent signaling pathways. In insects, the hormones ecdysone and juvenile hormone (JH) control the timing of the metamorphic transition and hence growth duration (1). The conserved insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR) pathways regulate growth rate (1). Recent evidence in *Drosophila melanogaster* indicates, however, that IIS and TOR signaling regulate ecdysone synthesis (2–5), whereas ecdysone antagonizes IIS (2, 6). This interaction between the mechanisms that regulate growth duration and those that control growth rate appears to coordinate the two processes, and may be a general feature of size regulation. To test this hypothesis, we explored whether JH also regulates growth rate in *Drosophila*.

In the tobacco hornworm *Manduca sexta*, JH regulates growth duration by regulating the hormonal response to critical weight, a size checkpoint used to determine when to end growth and begin metamorphosis (7). A decline in circulating JH initiates the first step in the hormonal cascade that begins with attainment of critical weight, and ends, after a terminal growth period (TGP), in the rise in circulating ecdysone that stops body growth (8–10). Starvation maintains high rates of JH synthesis in the JH-producing tissue, the corpora allata (CA) (9), and delays the critical weight transition (8). Removal of the CA (CAX) causes larvae to reach critical weight earlier than normal and at a smaller size (8, 11). Application of JH suppresses the critical weight transition and delays metamorphosis, resulting in larger size (8).

Intriguingly, recent studies show that, like CAX *Manduca*, CAX *Drosophila* larvae are smaller than normal (12, 13). How JH regulates body size in *Drosophila* is, however, unknown. Like *Manduca*, the cessation of growth in *Drosophila* is associated with attainment of critical weight in the final instar (14–16) followed by a TGP (17). Unlike *Manduca*, however, feeding JH to *Drosophila* larvae only delays the timing of metamorphosis at very high concentrations (18), suggesting that JH does not regulate the critical weight transition in this species. Finally, larval application of JH causes decreased adult size in *Drosophila* (19), opposite the expected effect if JH functions as it does in lepidopterans.

Here we elucidate how JH influences body size in *Drosophila*. Surprisingly, we show that JH does not regulate body size by influencing critical weight. Rather, CAX larvae are smaller due to reduced growth rate. We further show that this reduction in growth rate is forkhead box O transcription factor (FOXO)-dependent, suggesting that JH may regulate IIS. Finally, we present evidence that the effect of JH on growth and final body size is mediated by ecdysone synthesis in the prothoracic gland. These data illustrate how the processes regulating developmental time are intertwined with those that regulate growth rate.

Results

Ablation of the Corpora Allata Does Not Affect Critical Weight but Retards Its Attainment. Previous studies indicated that ablation of the CA, and hence elimination of JH, reduces body size in *Drosophila* (12, 13). To explore whether this effect is mediated by critical weight, we measured critical weight in both CAX and sibling control larvae. Attainment of critical weight is marked by a change in the developmental response to starvation. Before attaining critical weight, starvation substantially delays pupariation

Significance

Understanding how organisms regulate their body size is a fundamental problem in biology. Body size regulation involves the careful integration of mechanisms that control growth rate with those that control growth duration. In insects, developmental hormones such as juvenile hormone and ecdysone regulate developmental transitions and growth duration. The conserved insulin-signaling pathway regulates growth rates. Our studies reveal an intimate link between the three, whereby juvenile hormone controls body size by regulating ecdysone synthesis, which in turn modifies insulin signaling. In vertebrates, hormones such as androgens and estrogens interact with insulin signaling to influence tumor growth. By studying the developmental context of hormone interactions, our data reveal fundamental features of body size regulation that have important consequences for understanding cancer growth.

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(15, 16). After attainment of critical weight, starvation actually accelerates the time to pupariation. We used this change in the starvation response of pupariation time to explore whether the absence of JH in CAX larvae affects critical weight and its timing.

Fig. 1*A* shows that ablation of the CA had no effect on critical weight. We used the breakpoint method to assay critical weight in CAX and control larvae (15, 20–22). This method exploits the fact that the relationship between larval weight at starvation and time to pupariation (TTP) changes at critical weight (Fig. 1*A*). We found that critical weight was not significantly different in CAX and control larvae (Fig. 1*B*): Critical weight was 0.71 mg in CAX larvae and 0.69 mg in control larvae. Allatectomy did delay when critical weight was attained: Critical weight was attained at 7.5 h after third-instar (L3) ecdysis (AL3E) in control larvae but at 15.9 h AL3E in CAX larvae (Fig. 1*C*).

In *Manduca*, starvation early in the final instar delays wing disc development (23). Removal of the CA allows wing disc development to proceed even in the absence of nutrition (23). Similarly, in *Drosophila*, patterning in the wing disc is delayed in starved precritical weight larvae (24). To test whether JH played a role in the repression of wing disc pattern in *Drosophila*, we examined whether the expression of Wingless (Wg) and Senseless (Sens) depended on nutrition in the wing imaginal discs of CAX larvae. Starving either CAX or control larvae of protein before critical weight (7 h AL3E) inhibited both Wg and Sens expression in their wing discs (Fig. S1 *A–D*). In contrast, starving CAX and control larvae after critical weight (15 h AL3E) did not (Fig. S1 *I–M*). When larvae are starved from 9 h AL3E, only 2 out of 15 CAX wing discs up-regulated Sens expression (Fig. S1 *E–H*) compared with 12 out of 18 control wing discs. This indicates that most CAX larvae have not attained critical weight by 9 h AL3E, whereas most control larvae have. Thus, ablation of the CA, and hence elimination of JH, appears to retard attainment of critical weight but does not affect critical weight itself nor the response of disc patterning to starvation.

Ablation of the Corpora Allata Shortens the Terminal Growth Period. Our data show that JH does not regulate body size by affecting critical weight in *Drosophila*. However, it is possible that CAX larvae are small because of a reduced TGP between attaining critical weight and the cessation of feeding. We therefore measured the time to pupariation from critical weight (TTP_{CW}) in fed and starved larvae as proxy for the TGP. Allatectomy did not influence the TTP_{CW} in starved larvae but did shorten the TTP_{CW} in fed larvae, albeit by only 6 h (Fig. 1*D*). Because of the delay in attaining critical weight, the total duration of the third larval instar showed only a slight, albeit significant, increase in CAX larvae (Fig. 1*C*). The effect of allatectomy on the TTP_{CW} of fed larvae is similar to the effect of starvation: Both starvation and allatectomy shorten the TTP_{CW} (Fig. 1*D*) (4, 15). Thus, genetic ablation of the CA appears to phenocopy starvation in this respect.

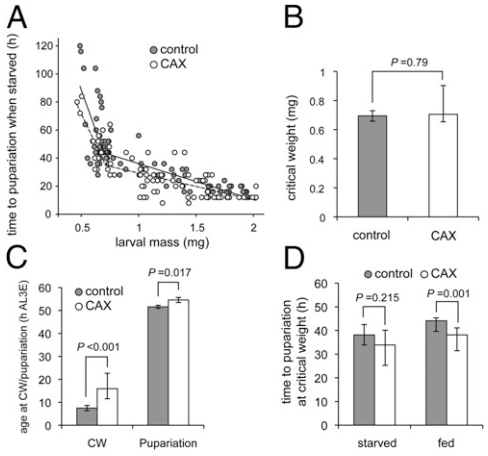

Fig. 1. Ablation of the CAX does not influence critical weight in *Drosophila*. (*A*) The time to pupariation for CAX and control larvae starved at different weights. The inflection in the regression line indicates the point at which larvae have reached critical weight. (*B*) Critical weight in CAX and control larvae is not significantly different (permutation test), as determined by a breakpoint analysis of the bisegmental regression line in *A*. (*C*) CAX larvae attain critical weight later than controls (permutation test), and pupariate slightly later (ANOVA). (*D*) Allatectomy does not appear to influence the time to pupariation from critical weight in starved larvae, but does shorten the TTP_{CW} in fed larvae (permutation test). Error bars are 95% confidence intervals. Sample sizes: permutation tests, $n = 73$ (CAX) and 97 (control); ANOVAs, $n = 150$ (CAX) and 390 (control).

Ablation of the Corpora Allata Slows Growth. Because ablation of the CA (*i*) had no effect on critical weight itself and (*ii*) did not appear to substantially reduce the TGP, it follows that CAX adults are smaller primarily due to a reduction in growth rate. To test this hypothesis, we measured larval growth from 0 to 30 h AL3E in CAX and sibling controls (Fig. 2). As expected, the growth rate of the CAX larvae was significantly slower than controls (Fig. 2). Further, CAX larvae were already significantly smaller immediately after molting to L3 (Fig. 2), suggesting that JH also affects either the growth rates or duration of the first and/or second instar.

To confirm that the slow growth of the CAX larvae was due to an absence of JH, we fed them a JH mimic, pyriproxyfen, from 0 h AL3E (Fig. 2). CAX larvae fed the JH mimic grew significantly faster than CAX larvae fed solvent (ethanol) alone, although still significantly slower than controls. Feeding a JH mimic to control larvae had no effect on their growth rate.

Ablation of the Corpora Allata Suppresses IIS. The observation that allatectomy reduces growth rate echoes the effects of reduced nutrition on larval development (4, 15, 16, 25). The major pathway by which nutrition influences development in *Drosophila*, and in all animals, is the IIS pathway. Importantly, the IIS pathway is also regulated by circulating hormones, particularly those involved in developmental transitions (e.g., ecdysone, androgens) (2, 6). We hypothesized, therefore, that the effect of JH on growth was mediated via the IIS pathway.

To test this hypothesis, we compared the expression of known components of the IIS cascade in CAX and control larvae using quantitative PCR. Suppression of IIS activates the transcription factor FOXO (26–28). FOXO then targets the expression of negative growth factors, such as *Thor*/*4E-binding protein* (*4E-BP*) (28). Also, FOXO drives the expression of *Insulin receptor* (*Inr*), which in turn represses FOXO activity, creating a negative feedback loop (28). Consequently, the expression of *Inr* and *4E-BP* can be used as proxies for IIS pathway activity (29).

In control larvae, expression of both *Inr* and *4E-BP* declined slightly at the beginning of the L3, but increased to a peak toward the end (Fig. 3 *A* and *B*). Ablation of the CA resulted in a significant increase in the expression of *Inr* and *4E-BP* between 0 and 32 h AL3E (Fig. 3 *C* and *D*), suggesting that insulin signaling is suppressed in CAX larvae. This early increase in *Inr* and *4E-BP* expression was not a consequence of a general increase in expression level: Expression of *Actin* was the same in CAX larvae and controls (Fig. S2*C*). After 32 h AL3E, *4E-BP* expression did not increase in CAX larvae as it did in controls, but *Inr* increased in both CAX and control larvae.

To further confirm that CAX larvae had suppressed insulin signaling, we used the *FOXO response element* (*FRE*)-*luc* reporter construct to assay FOXO activity in CAX and control larvae. The construct contains the firefly luciferase gene under the transcriptional control of the Herpes simplex minimal promoter and eight direct repeats of the FRE (30). FOXO activity can therefore be assayed by measuring luciferase activity. FOXO activity was significantly higher in CAX larvae compared with controls (Fig. 3*E*), confirming that CAX larvae have suppressed IIS.

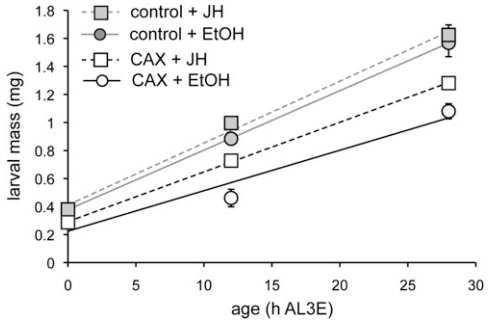

Fig. 2. Allatectomized (CAX) larvae grow more slowly than controls. Growth in CAX larvae is significantly slower than in control larvae ($ANCOVA_{genotype*age}$, $P < 0.001$). CAX larvae are significantly smaller than controls at ecdysis to the third instar (*t* test, $P < 0.001$). The addition of pyriproxyfen, a JH mimic, to the food increases growth rate in CAX larvae ($ANCOVA_{treatment*age}$, $P = 0.0023$), although not to the same rate as controls ($ANCOVA_{treatment*age}$, $P = 0.0103$). Pyriproxyfen has no effect on the growth rate of control larvae ($ANCOVA_{treatment*age}$, $P = 0.5972$). Error bars are 95% confidence intervals and are obscured by the data points in some cases. Lines are from linear regression. EtOH, ethanol. Sample sizes: ANCOVAs, $n = 36$ (CAX + EtOH), 42 (CAX + JH), 41 (control + EtOH), and 43 (control + JH); *t* test, $n = 16$ (CAX) and 14 (control).

FOXO Is Necessary for the Size Reduction in CAX Flies. To test whether the effect of allatectomy on growth rate is FOXO-dependent, we ablated the CA in flies mutant for *FOXO*. Allatectomy reduced final body size in animals that were wild-type for *FOXO*, but did not significantly affect final body size in flies mutant for *FOXO* (Fig. 3*G*). Thus, *FOXO* is necessary for the size reduction in CAX flies, suggesting that the effect of JH on growth depends on the IIS pathway.

Ablation of the Corpora Allata Elevates Ecdysone Signaling. In *Drosophila*, elevated ecdysone synthesis by the prothoracic gland (PG) can reduce growth rate by suppressing systemic IIS, without affecting developmental timing (2). Further, there is evidence that JH can suppress ecdysone synthesis by the PG in vitro (31). One hypothesis, therefore, is that loss of JH reduces growth rate by derepressing ecdysone synthesis, elevating the ecdysone titer, and suppressing systemic IIS. To test this, we measured ecdysone titers in control and CAX larvae and found that, consistent with our hypothesis, CAX larvae had significantly elevated ecdysone levels (Fig. 3*F*). We also examined the expression of the B isoform of the *ecdysone-induced protein 74EF* (*E74B*) an ecdysone-response gene commonly used as an indicator of ecdysone signaling, and found that it was also elevated in CAX larvae relative to controls (Fig. S2 *A* and *B*). Overall, there was a positive relationship between *Inr*/*4E-BP* expression and *E74B* expression throughout larval development when controlling for larval age and phenotype (Fig. S2 *D* and *E*), supporting the previous observation that ecdysone signaling negatively regulates IIS.

Knockdown of Met Systemically and in the PG Alone Reduces Final Body Size. Like CAX larvae, mutant larvae lacking both of the duplicated, putative JH receptor genes *Methoprene-tolerant* (*Met*) and *germ cell-expressed* (*gce*) (32) grow slowly (Fig. S3). In this case, feeding these larvae the JH mimic pyriproxyfen did not rescue growth to normal rates. Loss of *Met* alone caused the formation of small pupae and adults, and these effects on size were rescued by ubiquitous expression of *Met* (Fig. 4*A*).

The observation that CAX larvae have elevated ecdysone signaling and reduced IIS is consistent with the hypothesis that JH regulates body size by controlling the synthesis and release of ecdysone. In *Manduca*, JH suppresses ecdysteroidogenesis by inhibiting the synthesis of prothoracicotropic hormone (PTTH) in the brain (8). In *Drosophila*, however, JH can act directly on the PG to suppress ecdysone synthesis, at least in vitro (31). To identify the tissue through which JH influences ecdysone synthesis, we disrupted JH signaling in individual tissues by knocking down the expression of *Met* with targeted RNAi. Knockdown of *Met* in the PTTH-producing neurons (*ptth>Met.RNAi*) or the entire nervous system (*elav>Met.RNAi*) had no effect on pupal size, whereas knockdown of *Met* in the PG (*phm>Met.RNAi*) significantly reduced pupal size (Fig. 4*B*). This reduction in pupal size was correlated with a significant reduction in growth rate from 0 to 25 h AL3 (Fig. 4*C*). Knocking down *Met* in the PG did not affect the duration of the L3 nor the minimal viable weight, a common proxy for critical weight (4, 15, 33, 34) (Fig. 4 *D* and *E*).

Discussion

Our results show that JH regulates body size in *Drosophila* not by controlling growth duration, as it does in other insects, but by regulating growth rates. The mechanism for this control is FOXO-dependent and appears to be through the JH regulation of ecdysone synthesis, an antagonist of IIS. Below we discuss our results in the context of what has been previously described for size regulation in *Manduca* and *Drosophila*.

Critical Weight: Variation in Mechanisms. For both *Manduca* and *Drosophila* larvae, attaining critical weight means that starvation

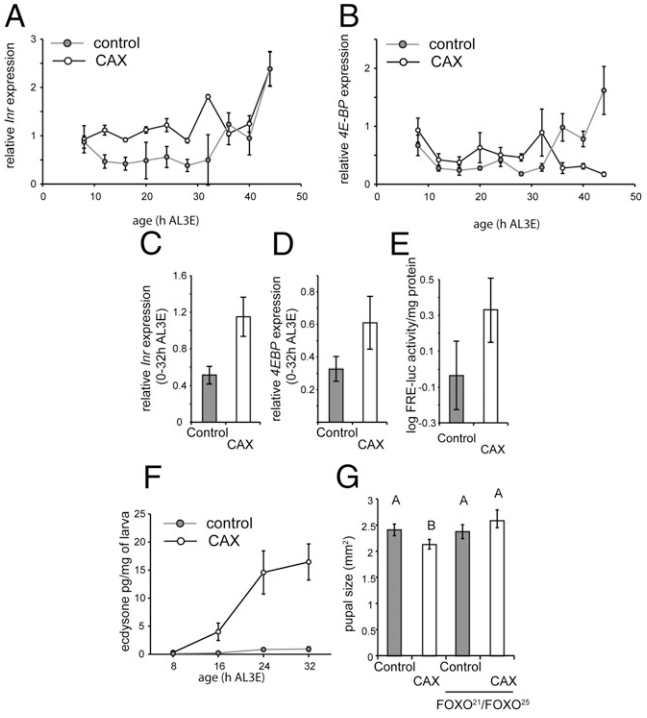

Fig. 3. Loss of JH down-regulates the activity of the IIS pathway and up-regulates ecdysone signaling. (*A* and *B*) The expression profile of *Inr* and *4E-BP* throughout larval development in CAX and control larvae ($n = 5$ biological replicates for each data point). (*C* and *D*) Expression of *Inr* and *4E-BP* is significantly up-regulated from 0 to 32 h AL3E in CAX larvae relative to control (ANOVA, $P < 0.001$ for both; $n = 35$ for CAX and control), consistent with a systemic decrease in insulin signaling and activation of FOXO. (*E*) FOXO activity is up-regulated in CAX larvae relative to control (ANOVA, $P = 0.02$; $n = 7$ for CAX and 6 for control). (*F*) Levels of ecdysone are significantly higher from 0 to 32 h AL3E in CAX larvae relative to controls [ANOVA, $P < 0.001$; five replicates per time point ($n = 20$) for both CAX and control]. (*G*) The effect of allatectomy on body size interacts significantly with the presence or absence of FOXO [ANOVA, $P < 0.001$; $n = 28$ (CAX), 19 (control), 8 (CAX – *FOXO*), 13 (control – *FOXO*)], such that allatectomy reduces final body size in genetically wild-type flies but not in flies mutant for *FOXO* [Tukey's Honest Significant Difference (HSD) test]; columns with different letters are significantly different at $P < 0.05$). Error bars are 95% confidence intervals.

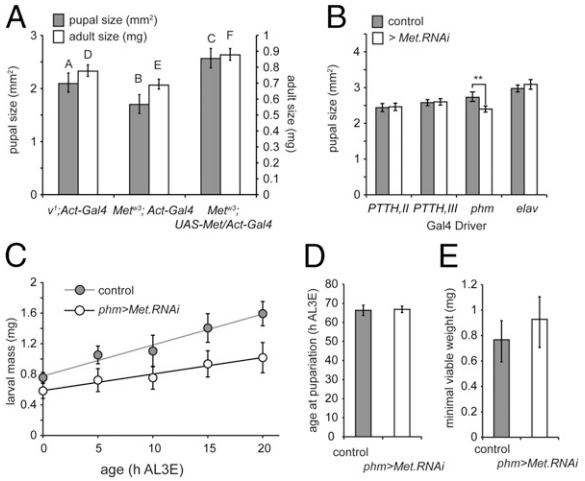

Fig. 4. JH acts on the prothoracic gland to regulate ecdysone synthesis and modify growth. (*A*) Mutations in the JH receptor *Met* reduce both mean pupal and adult body size, and these reductions can be rescued by ubiquitous overexpression of *Met* [ANOVA, $P < 0.001$; $n = 30$ (v^1; *Act-GAL4*), 41 (Met^{w3}; *Act-GAL4*), 29 (Met^{w3}; *UAS-Met/Act-GAL4*), Tukey HSD; columns with different letters are significantly different at $P < 0.01$]. (*B*) There is a significant reduction in body size when *Met* is knocked-down in the PG [*t* test, $P < 0.001$; $n = 20$ (*phm>GFP*), 20 (*phm>Met.RNAi*)], but not when *Met* is knocked down in the PTTH-producing cells or the central nervous system [*t* test, $P > 0.05$ for both; $n = 24$ (*PTTH,II>GFP*), 25 (*PTTH,II>Met.RNAi*), 26 (*PTTH, III>GFP*), 26 (*PTTH,III>Met.RNAi*), 22 (*elav>GFP*), 23 (*elav>Met.RNAi*)]. $^{**}P < 0.001$. (*C–E*) Reducing JH signaling in the PG reduces larval growth rates ($ANCOVA_{genotype*time}$, $P = 0.0113$; $n = 214$), but does not alter development time [*t* test, $P = 0.637$, $n = 83$ (*phm>GFP*), 136 (*phm>Met.RNAi*)] or minimal viable weight [nominal logistic regression, $P = 0.196$; $n = 69$ (*phm>GFP*), 70 (*phm>Met.RNAi*)]. Lines are from linear regression. Error bars are 95% confidence intervals.

no longer delays metamorphosis (4, 14, 16, 35). However, several differences exist in the critical weight of these two insects. First, after *Manduca* larvae reach critical weight, starvation has no effect on the time to metamorphosis (35). In contrast, when postcritical weight *Drosophila* larvae are starved, they accelerate their time to metamorphosis (4, 15). In addition, *Manduca* larvae reach critical weight ~50% into their final instar (35). *Drosophila* larvae reach critical weight earlier, ~25% into their final instar (24). These differences suggest that the mechanisms regulating critical weight are not identical in the two species.

The physiology of critical weight supports this notion. *Manduca* larvae allatectomized immediately after the molt to the final instar, then starved, enter metamorphosis 1–2 d earlier than starved sham-operated controls (8). This occurs because JH can no longer suppress the release of PTTH, which stimulates ecdysone release and wandering 1.5 d after larvae reach critical weight (8, 11). Application of JH delays PTTH release and wandering (8). However, infusion of ecdysone into precritical weight larvae cannot promote premature metamorphosis in final-instar *Manduca* larvae (8). These classic experiments suggest that the decline of JH in the final instar of *Manduca* is the primary response to the developmental transition at critical weight (8, 10, 36).

In *Drosophila*, our data show that JH does not affect critical weight. Rather, previous work suggests that the critical weight transition occurs as a result of an increase in the ecdysone titer early in the L3 stimulated by IIS/TOR signaling in the PG(2–4,24). Thus, where JH appears to regulate critical weight in *Manduca*, ecdysone appears to regulate critical weight in *Drosophila*. The significance of this difference in regulation is not yet clear.

Nevertheless, although JH does not appear to regulate critical weight in *Drosophila*, our data suggest that it does influence ecdysone synthesis. This presents something of a paradox: If JH regulates ecdysone, and ecdysone regulates critical weight, then loss of JH should also affect critical weight. One solution to this paradox is the observation that a moderate change in ecdysone signaling can affect IIS without affecting developmental timing (2, 22). JH may affect the basal levels of ecdysone synthesized by the PG but not influence the timing of ecdysone peaks that coincide with the attainment of critical weight, larval wandering, and pupariation (22).

Juvenile Hormone Regulation of Growth Rates. Our research indicates that JH regulates growth in *Drosophila* and that this regulation is dependent on FOXO, a key effector of the IIS pathway. These data support and extend previous studies that indicate crosstalk between JH and IIS in several holometabolous insects (37).

In *Manduca* larvae, JH acts to regulate the growth of the imaginal discs in response to changes in IIS (23). As in *Drosophila*, starving *Manduca* larvae before attainment of critical weight suppresses growth and development of their imaginal discs. Ablation of the CA overrides these effects so that disc growth and development continue even in the absence of nutrition (23). Similarly, although imaginal discs continue to develop when cultured in a hormone-free but nutrient-rich medium, development is suppressed when JH is added to the medium (38, 39). The growth-suppressing capacity of JH is overridden when insulin is also added to the medium (38). Thus, JH appears to regulate growth and development of the imaginal discs by sensitizing them to changes in IIS: In the absence of JH, IIS is not necessary for growth and development, whereas in the presence of JH, it is. The same phenomenon does not appear to be acting in *Drosophila*. In this case, starvation of precritical weight larvae suppressed imaginal disc growth and development, regardless of whether or not they had the CA and thus JH.

In adult *Drosophila*, females with hypomorphic mutations of the insulin receptor suppress vitellogenesis (39). The effects of suppressed IIS can be reversed through application of methoprene, suggesting that IIS regulates JH synthesis (39). Subsequent studies have demonstrated that IIS regulates JH in the CA of developing larvae. Suppression of IIS in the CA alone is sufficient to inhibit expression of 3-hydroxy-3-methylglutaryl CoA reductase, an enzyme involved in cholesterol and JH biosynthesis (40). The result is a reduction in final body size, genocopying the effects of genetic allatectomy (12) and the Met^{W3} null mutation (41, 42). Intriguingly, our data suggest that insulin signaling is also downstream of JH, by demonstrating that the reduction in body size in CAX larvae correlates with activation of FOXO and is FOXO-dependent. Thus, JH and IIS appear to interact through a positive feedback loop: A reduction in IIS in the CA suppresses JH synthesis, which in turn reduces systemic IIS.

Positive feedback loops are relatively unusual in physiological systems, in part because they tend to cause system instability. The function of the positive feedback loop between JH and IIS is unclear, but may serve to rapidly reduce the level of circulating JH at a particular point in development by suppressing its IIS-regulated synthesis. This relationship between JH and IIS is in contrast to the relationship between ecdysone and IIS, where the interaction forms a negative feedback loop: IIS in the PG promotes ecdysteroidogenesis (2–4), which in turn suppresses systemic IIS and reduces growth rate (6). The effects of ecdysone on growth rates are bidirectional, such that an increase in ecdysone synthesis decreases growth rate, whereas a decrease in ecdysone synthesis increases growth rate (2, 4). In contrast, the influence of JH on growth rates is unidirectional, such that only loss of JH appears to have an effect, whereas addition of JH does not. This may be because JH is not limiting for IIS under normal physiological conditions, and so feeding larvae pyriproxyfen does not further increase growth rate.

Although our data are consistent with the hypothesis that JH regulates growth via the IIS pathway, it is possible that other mechanisms suppress growth via FOXO, followed by subsequent adjustment in IIS components. For example, FOXO activity is

positively regulated by the stress-inducible kinases Jun N-terminal kinase (JNK) and STE20-like protein kinase 1 (MST1) (43). Consequently, ablation of the CA may suppress growth via activation of the JNK- or MST1/2-signaling pathways. However, our data also implicate ecdysone in the JH regulation of growth, which is a known antagonist of IIS. Ecdysone synthesis has been shown to negatively regulate IIS throughout the body through its action on the fat body (2, 6), and JH can inhibit ecdysone synthesis by the PG in vitro in *Drosophila* (31). Our data connect these two previously unrelated observations and suggest that JH acts in vivo in *Drosophila* to regulate systemic IIS and organismal growth by controlling ecdysone synthesis. The significance of the JH regulation of ecdysone and IIS during normal *Drosophila* development requires further elucidation.

Conclusions

Collectively, our data support the hypothesis that there is an intimate link between the processes that regulate developmental transitions, such as puberty and metamorphosis, and the processes that regulate growth. This link provides a physiological context for the observation that, in humans, developmental hormones such as androgens and estrogens also drive growth of several forms of cancer (44, 45) as well as benign tumors such as vascular malformations (46). The observation that both JH and ecdysone regulate growth in a FOXO- and IIS-dependent manner suggests that IIS may be the nexus at which the hormonal regulation of growth rate, growth duration, and developmental transition meets. Studies of such developmental processes may therefore provide key insights into the growth-regulatory pathways that are targeted in hormone-driven cancers.

Materials and Methods

Fly Stocks and Larval Rearing Conditions. The following flies were used in this study: *Aug-21* (47); *UAS-grim* (48); *UAS-reaper* (Bloomington *Drosophila* Stock Center; 5824); *FRE-Luc* (30); *FOXO*^{21} and *FOXO*^{25} (49); *Met*^{W3} (42); *Met*^{27},*gce*$^{2.5k}$ (32); *UAS-Met* and the progenitor stock (41); *v; UAS-Met.RNAi* [Vienna Drosophila RNAi Center (VDRC) 45852] and the progenitor stock (VDRC; 6000); *phm-GAL4* (4); e*lav-GAL4* (Bloomington *Drosophila* Stock Center; 8760); and *ptthII-GAL4* and *ptthIII-GAL4* (33). All flies were reared at low density on standard cornmeal molasses fly medium at 29 °C as described previously (4, 24), unless otherwise stated.

Ablation of the Corpora Allata. To genetically ablate the CA, we crossed the *w; Aug21/CyO actin-GFP* fly line (47) with *w; UAS-grim*. The CAX larvae (*Aug21; UAS-grim*) were separated from their sibling controls (*CyO actin-GFP; UAS-grim*) using the absence of GFP. We combined these alleles with *FRE-luc* to assay FOXO activity in CAX and control larvae, described below. We generated CAX FOXO nulls by crossing *w; Aug21/CyO actin-GFP; FOXO25/TM6B* with *w; UAS-rpr/CyO actin-GFP; FOXO21/TM6B*. Unlike *Aug21>grim* larvae (12), 5% of the *Aug21>rpr* larvae eclosed as adults. Out of 389 pupae from a cross between *w; Aug21/CyO actin-GFP* and *UAS-rpr*, 205 eclosing adults were CyO and 10 were not. Nevertheless, when we dissected 20 *Aug21>rpr* white prepupae none had CA, so these larvae are primarily CAX. Similarly, 17 dissected *Aug21>rpr FOXO25/FOXO21* white prepupae also did not have CA. *FOXO25/FOXO21* transheterozygotes produce no detectable protein (50) and are assumed to be nulls (49).

Larval Weight and Pharate Adult Size Measurements. For larval weight, we individually weighed larvae using a Mettler Toledo XP2U Ultra-microbalance [readibility (*d*) 0.1 μg] or XP26 Microbalance (*d*, 1 μg). To compare growth rates, we collected newly molted L3 larvae every 2 h. These larvae were then returned to food and then weighed at the desired age. For the JH rescue experiments, newly molted larvae were fed a JH mimic by adding 5 μg pyriproxyfen diluted in 50 μL ethanol to 5 mL fly medium (1 ppm pyriproxyfen) as described previously (12). Control larvae were fed 50 μL ethanol added to 5 mL fly medium.

We calculated growth rates by regressing larval weight against age, and compared among genotypes and hormone treatments by testing for an interaction between age and genotype or treatment using an analysis of covariance (ANCOVA). We measured pharate adult body size either by weighing the pupae or by measuring their total area in the coronal plane (51).

Critical Weight and Developmental Timing. Individual CAX and control (GFP-positive) L3 larvae were weighed and placed in a 1.5-mL microtube with a 10 × 50 mm strip of moist Kimwipe. TTP was recorded by checking larvae every 4 h and, if pupariated, larvae were weighed again 24 h later.

We used the relationship between larval weight and TTP to find the critical weight, using the breakpoint method as described previously (15, 20–22). Briefly, the breakpoint method exploits the fact that the relationship between larval mass at starvation and TTP changes at critical weight, and this change can be identified using a bisegmental linear regression. We repeated the analysis on 1,000 bootstrap samples to generate 95% confidence intervals for the critical weight and the TTP_{CW} when starved for CAX and control larvae. We used a permutation test with 1,000 replicates to generate a null distribution of the difference in critical weight and TTP_{CW} in CAX and control larvae, and used this distribution to estimate a *P* value for the observed differences.

To measure the mean duration of the L3, we collected 4-h cohorts of ecdysing L3 larvae and checked for pupariation every 8 h. The experiment was replicated four times and the mean duration of the L3 was calculated using a mixed-effect ANOVA, with larval genotype as a fixed effect and replicate as a random effect.

To estimate the TTP_{CW} for fed larvae, we first calculated the time at which larvae attained critical weight by fitting our value for critical weight to the larval growth curve. We then subtracted this from our calculation of the mean duration of the L3. We repeated the analysis on 1,000 bootstrap samples to generate 95% confidence intervals for the TGP in CAX and control larvae, and used a permutation test to estimate a *P* value for the observed differences.

Protein Starvation and Immunocytochemistry. To determine the effects of JH on the patterning of wing discs from protein-starved larvae, we transferred 10–15 larvae at 7, 9, or 15 h AL3E to a 20% (wt/vol) sucrose solution. We dissected 10–15 larvae immediately for stage controls and returned 10–15 larvae to fly medium as fed controls. Protein-starved and -fed larvae were dissected 24–25 h later and fixed using 4% (vol/vol) paraformaldehyde in PBS for 30 min at room temperature. Samples were processed for immunocytochemistry as described previously (24) using a 1:100 dilution of mouse anti-Wingless (concentrate from the Developmental Studies Hybridoma Bank) and a 1:1,000 dilution of guinea pig anti-Senseless antibody (from Hugo Bellen, Baylor College of Medicine, Houston). We imaged samples using a Leica LSM 510 or 710 multiphoton microscope.

Quantitative PCR. We used two-step quantitative real-time PCR to assay the expression of *Inr*, *4E-BP*, and *E74B* during the L3 in CAX and control larvae. Larvae were staged into 4-h cohorts at ecdysis to the L3. We sampled ∼25 larvae every 4 h from 8 to 44 h AL3E, and divided larvae from each time point into five biological replicates, each comprising four or five larvae. RNA was extracted using an RNeasy Mini Kit (Qiagen) and reverse-transcribed to cDNA using the GoScript Reverse Transcription System (Promega), and transcript levels were assayed using GoTaq Green Master Mix (Promega) using a standard curve and normalized against expression of 28S. Primers are listed in Table S1. Standard curves were generated using seven serial dilutions of total RNA extracted from two first-instar larvae, two second-instar larvae, two L3 larvae (male), two pupae (male), and two adult flies (male) of OregonR. Differences in gene expression between CAX and control larvae in the first 32 h AL3E were analyzed using an ANOVA ($Y_{ik} = u + G_j + A_k + e_{ijk}$, where *Y* is expression, *u* is mean expression, *G* is genotype, *A* is age, and *e* is error), treating both age and genotype as categorical variables.

***FRE-Luciferase* Assays.** We crossed *FRE-luc/In(2LR)Gla, wg Gla-1 Bc1; UAS-Grim/TM6B* with *Aug21/CyO tub-GFP* and compared FOXO activity in *FRE-luc/Aug21; UAS-grim* CAX larvae with FOXO activity in *FRE-luc/CyO tub-GFP; UAS-grim* control larvae. We staged larvae into 2-h cohorts at ecdysis to the L3. They were allowed to feed for an additional 24 h and then removed from the food, washed, and stored at −80 °C. Larvae were divided into three or four replicates of three larvae, homogenized in 200 μL PBS with protease inhibitor, and centrifuged at 15,900 × *g* for 5 min. We then tested 10 mL of the supernatant for luciferase activity using the Promega Luciferase Assay System. We measured the protein concentration for each sample using a standard BCA assay (Quantipro BCA Assay Kit, Sigma-Aldrich, St. Louis) and normalized the luciferase activity as activity per milligram. We repeated the experiment over two trials and calculated the mean (log) luciferase activity across trials using an ANOVA.

Ecdysone Quantification. Carefully staged larvae were washed twice in distilled water, weighed, and then flash-frozen on dry ice. Larvae were preserved

in three times their volume of ice-cold methanol and kept at −80 °C. To process the samples, we first homogenized the tissue and centrifuged samples at 15,700 × *g* at 4 °C. The supernatant was transferred into new tubes and the methanol was evaporated off in a vacuumed centrifuge (Savant SVC-100H with a RH 40-11 rotor). Ecdysone concentration was quantified using the Cayman Chemical 20-Hydroxyecdysone EIA Kit according to the manufacturer's instructions. Differences in ecdysone concentration between CAX and control larvae in the first 32 h AL3E were analyzed using an ANOVA ($Y_{ik} = u + G_j + A_k + e_{ijk}$, where Y is ecdysone concentration, u is mean ecdysone concentration, G is genotype, A is age, and e is error), treating both age and genotype as categorical variables.

***Met* Mutant Analysis.** We compared pharate adult size of Met^{W3}*; Act-Gal4*, Met^{W3}*; Act-Gal4/UAS-Met*, and *v; Act-Gal4* (42) as described above. We knocked down expression of *Met* in specific tissues using *UAS-Met.RNAi* (VDRC; 45852) combined with *phm-GAL4* (PG), e*lav-GAL4* (nervous system), and *ptthII-GAL4* and *ptthIII* (PTTH-producing neurons). Coisogenic control larvae were generated from the RNAi progenitor stock (VDRC; 60000) combined with the GAL4 lines. We used minimal viable weight for pupariation [MVW(P)] as a proxy for critical weight for *phm>Met.RNAi* and controls (4, 15, 33, 34). MVW(P) is defined as the minimal weight at which 50% of larvae survive to pupariation when starved. We used a nominal logistic regression to predict the weight at which 50% of the starved larvae survive to pupariation. Larvae were reared at 25 °C.

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